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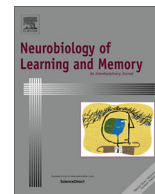
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Review

Generation of multi-innervated dendritic spines as a novel mechanism of long-term memory formation

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ABSTRACT

NMDA receptor-dependent long-term potentiation (LTP) at hippocampal CA1 synapses is a well-accepted mechanism underlying long-term memory (LTM) formation. However, studies with mice that lack threonine-286 autophosphorylation of α CaMKII have shown that hippocampal LTM can be formed despite absence of NMDA receptor-dependent CA1 LTP. After multiple training trials, LTM formation in these mutants is linked to the generation of multi-innervated dendritic spines (MIS), a spine that receives typically two presynaptic inputs. PSD-95 overexpression is sufficient for MIS generation and depends on mTOR signaling. LTM that involves MIS generation appears less modifiable upon retrieval in comparison to LTM without MIS generation. Taken together, MIS generation appears to be a novel LTM mechanism after multiple training trials, which may occur in diseases with impaired LTP or conditions affecting negative feedback CaMKII signaling at the synapse.

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1. In search of cellular mechanisms of long-term memory formation

Mechanistic understanding of long-term memory (LTM) is one of the big challenges in neuroscience. As a cellular mechanism underlying LTM, NMDA receptor-dependent long-term potentiation (LTP) has received a lot of attention. This type of plasticity occurs at hippocampal CA1 synapses after training in relevant memory tasks (Gruart, Muñoz, & Delgado-García, 2006; Whitlock, Heynen, Shuler, & Bear, 2006). Further, pharmacologic or molecular biological alteration of NMDA receptor-dependent LTP at CA1 synapses affects LTM formation or storage (Giese, 2012; Martin, Grimwood, & Morris, 2000; Silva, 2003). Recently, the causal link between NMDA receptor-dependent LTP and LTM has been strengthened with an optogenetic approach, where long-term depression (LTD) impairs LTM and LTP induction is sufficient to restore LTM (Nabavi et al., 2014). Whilst this evidence demonstrates that NMDA receptor-dependent LTP is important for LTM, it has also become clear that there is more to LTM than just NMDA receptor-dependent LTP. In particular, investigations with the α CaMKII^{T286A} mouse model have shown that LTM can be

formed in the absence of NMDA receptor-dependent LTP at CA1 synapses.

2. The α CaMKII^{T286A} mouse model

The α -isoform of calcium/calmodulin-dependent kinase II (α CaMKII) is the most abundant signaling protein in the postsynaptic density of glutamatergic neurons in the forebrain (Kennedy, 2000). NMDA receptor opening at the postsynaptic membrane leads to activation of α CaMKII. Once activated, α CaMKII autophosphorylates at the so-called autonomy site at threonine-286 (T286). This autophosphorylation prolongs α CaMKII activity for about one minute after LTP induction (Lee, Escobedo-Lozoya, Szatmari, & Yasuda, 2009). α CaMKII^{T286A} mice have a targeted point mutation in the α CaMKII gene that prevents phosphorylation at the autonomy site threonine-286 (Giese, Fedorov, Filipkowski, & Silva, 1998). Thorough electrophysiological investigation have established that the lack of T286 autophosphorylation completely abolishes NMDA receptor-dependent LTP at hippocampal CA1 synapses in adult, but not at neonatal age (Cooke et al., 2006; Giese et al., 1998; Irvine et al., 2011; Radwanska et al., 2011; Villers, Giese, & Ris, 2014; Yasuda, Barth, Stellwagen, & Malenka, 2003). These LTP impairments were demonstrated in hippocampal slices and in anaesthetised mice. However, despite the absence of NMDA receptor-dependent LTP at CA1 synapses, α CaMKII^{T286A} mice can

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form hippocampus-dependent LTM (Irvine, Vernon, & Giese, 2005; Irvine et al., 2011; Radwanska et al., 2011). This was shown after training in contextual fear conditioning and the passive avoidance task. The lack of T286 autophosphorylation abolishes one-trial LTM formation, but LTM can be formed with massed training. For example, five massed conditioning trials enable contextual fear LTM formation in $\alpha\text{CaMKII}^{\text{T286A}}$ mice (Irvine et al., 2005, 2011; Radwanska et al., 2011). Importantly, this contextual fear LTM in the absence of T286 autophosphorylation depends on the hippocampus as revealed by post-training lesion experiments (Irvine et al., 2011). Further, immunohistochemical analysis after contextual fear conditioning showed that the $\alpha\text{CaMKII}^{\text{T286A}}$ mutants do not upregulate Zif268 and NR4a1 in hippocampal area CA1 (Radwanska et al., 2011), immediate-early genes that are required for the late phase of NMDA receptor-dependent LTP (Bridi & Abel, 2013; Jones et al., 2001). Thus, the deficient upregulation of these immediate-early genes indicates that contextual fear conditioning-induced NMDA receptor-dependent LTP is impaired in the $\alpha\text{CaMKII}^{\text{T286A}}$ mutants. Moreover, immediate-early gene imaging after contextual fear conditioning showed that there is no upregulation in alternate brain regions in the mutants, demonstrating that there is no compensation at the systems level that leads to contextual fear LTM in $\alpha\text{CaMKII}^{\text{T286A}}$ mice (Radwanska et al., 2011). Taken together, this concludes that in $\alpha\text{CaMKII}^{\text{T286A}}$ mice hippocampus-dependent LTM can be formed despite complete lack of NMDA receptor-dependent LTP at CA1 synapses. An alternate plasticity mechanism at CA1 synapses appears sufficient for LTM formation in $\alpha\text{CaMKII}^{\text{T286A}}$ mice.

LTP involves the strengthening of existing synapses. In principle, it is conceivable that synaptogenesis may compensate for impaired LTP to enable LTM formation in the $\alpha\text{CaMKII}^{\text{T286A}}$ mice. This idea was tested with electron microscopy (EM) analysis (Radwanska et al., 2011). Excitatory synapse numbers in stratum radiatum of area CA1 were compared: (i) before, and at (ii) 2 h and (iii) 24 h after contextual fear conditioning. Both wild-type and $\alpha\text{CaMKII}^{\text{T286A}}$ mice showed up-regulated synapse numbers at 2 h after conditioning. This synaptogenesis occurred in the mutants despite impaired induction of the immediate-early genes c-Fos, Zif268 and NR4a1 (Radwanska et al., 2011). Thus, local protein synthesis might lead to contextual fear conditioning-induced synaptogenesis (Holt & Schuman, 2013). The contextual fear conditioning-induced increase in synapse numbers is transient as after 24 h there is not net increase in synapse numbers (Radwanska et al., 2011). This finding appears in contradiction with a dendritic spine analysis using Golgi staining, showing increased spine density in hippocampal area CA1 24 h after contextual fear conditioning (Restivo, Vetere, Bontempi, & Ammassari-Teule, 2009). However, this study analyzed spines after a retrieval trial that triggers reconsolidation and which could have induced spinogenesis.

Transient up-regulation in synaptogenesis cannot account for LTM in $\alpha\text{CaMKII}^{\text{T286A}}$ mice, which was tested much later than 2 h after conditioning (Irvine et al., 2005). However, further EM data analysis revealed that generation of an atypical excitatory synapse type, multi-innervated dendritic spines (MIS) (Fig. 1), correlated specifically with LTM formation in $\alpha\text{CaMKII}^{\text{T286A}}$ mice and not in wild-type mice (Radwanska et al., 2011).

3. Multi-innervated dendritic spines and long-term memory formation

The vast majority of excitatory synapses in hippocampal area CA1 have a 1:1 relationship between a presynaptic terminal and a dendritic spine. However, in some cases a dendritic spine receives more than one presynaptic input (Fiala, Feinberg, Popov, & Harris, 1998). These structures as shown in Fig. 1 have been

termed multi-innervated dendritic spines (MIS) (Nikonenko, Jourdain, & Muller, 2003). In adult hippocampal area CA1, MIS have typically two presynaptic inputs and they represent less than 1% of excitatory synapses (Petrak, Harris, & Kirov, 2005; Radwanska et al., 2011). MIS develop by the attraction of presynaptic terminal(s) onto an existing dendritic spines (Nikonenko et al., 2008; Petrak et al., 2005). In hippocampal slice cultures NMDA receptor activation can induce MIS generation (Nikonenko et al., 2003). Further, PSD-95 overexpression is sufficient to generate MIS (Nikonenko et al., 2008). This overexpression leads to binding of PSD-95 to neuronal nitric oxide synthase (nNOS), which causes production of the retrograde messenger nitric oxide that attracts presynaptic terminal(s) onto the existing dendritic spine with multiple distinct postsynaptic densities (PSDs) (Nikonenko et al., 2008).

Contextual fear conditioning with $\alpha\text{CaMKII}^{\text{T286A}}$ mice showed for the first time that behavioral experience can induce MIS generation (Radwanska et al., 2011). Training-induced MIS generation was analyzed only in hippocampal area CA1. It remains possible that MIS generation also occurs in other brain areas, such as the amygdala, after contextual fear conditioning of $\alpha\text{CaMKII}^{\text{T286A}}$ mice. As in hippocampal slice culture experiments, the behaviorally-induced MIS generation in CA1 correlates with PSD-95 protein expression (Radwanska et al., 2011). PSD-95 up-regulation occurs in $\alpha\text{CaMKII}^{\text{T286A}}$ mice after five massed conditioning trials, but not after a single conditioning trial. Further, PSD-95 up-regulation and the contextual fear LTM in $\alpha\text{CaMKII}^{\text{T286A}}$ mice depend on mTOR signaling (Radwanska et al., 2011). Taken together, this suggests that MIS generation enables contextual fear LTM formation in $\alpha\text{CaMKII}^{\text{T286A}}$ mice. Moreover, in normal wild-type mice MIS generation appears suppressed due to signaling induced by αCaMKII autophosphorylation.

Intriguingly, the identification of MIS generation as a LTM mechanism further underlines the importance of LTP as a LTM mechanism, as the generation of MIS can be considered as a kind of 'morphological LTP'. However, in contrast to 'chemical LTP' this 'morphological LTP' may be less modifiable as a multitude of molecular processes would have to be involved versus simple AMPA receptor endocytosis in the case of 'chemical LTP'. Thus, the question arises whether LTM, which involves MIS generation, has the same properties as LTM in wild-type mice. To this end, retrieval-induced modulation of contextual fear LTM in $\alpha\text{CaMKII}^{\text{T286A}}$ mice was studied (Radwanska et al., 2011). Retrieval can induce a destabilization process that is compensated by a protein synthesis-dependent reconsolidation process (Nader & Einarsson, 2010). These retrieval-induced processes are thought to be important for memory maintenance and memory updating. Memory destabilization can be assessed by pharmacologically blocking protein synthesis-dependent reconsolidation so that retrieval only induces destabilization that should erase the memory. However, if retrieval does not lead to memory erasure when reconsolidation is blocked, then the mechanisms of memory destabilization are impaired. Destabilization of contextual fear LTM after retrieval was found to be impaired in $\alpha\text{CaMKII}^{\text{T286A}}$ mice in contrast to normal wild-type mice. Whilst this indicates that T286 autophosphorylation is required for retrieval-induced LTM destabilization, it leaves the possibility that MIS generation forms a LTM that is less modifiable than LTM that does not involve MIS.

4. Conclusion and outstanding questions

The $\alpha\text{CaMKII}^{\text{T286A}}$ mice have been a great tool for identification of a novel LTM mechanism in the absence of NMDA receptor-dependent LTP at CA1 synapses. Studies with these mutants suggest that MIS generation is sufficient for LTM formation when LTP is absent. MIS generation requires multiple, massed

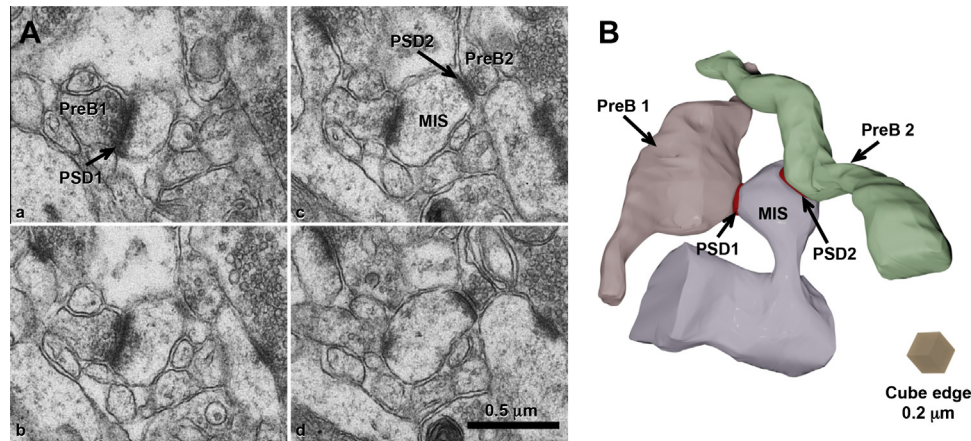


Fig. 1. Example of a multi-innervated dendritic spine (MIS) in hippocampal area CA1 of wild-type mice. A. Serial electron microscopy images of a MIS making contacts with two presynaptic boutons. B. 3D reconstruction of the MIS from the series shown in panel A. PreB, presynaptic bouton; PSD, postsynaptic density.

training, as a single training trial is not sufficient. MIS generation was found after contextual fear conditioning of $\alpha\text{CaMKII}^{\text{T286A}}$ mice (Radwanska et al., 2011). It is expected that MIS generation also enables LTM formation after training in other hippocampus-dependent memory tasks such as passive avoidance (see, Irvine et al., 2005). However, training of $\alpha\text{CaMKII}^{\text{T286A}}$ mice in the Morris water maze may not lead to MIS generation in the hippocampus, because even after very intensive training (12 trials per day) the mutants cannot form a spatial memory.

Based on our contextual fear conditioning experiments, LTM that involves MIS generation might not be destabilized upon retrieval impairing LTM updating. The signaling pathways underlying the crosstalk between LTP and MIS generation are illustrated in Fig. 2. In wild-type mice after NMDA receptor activation T286 autophosphorylation of αCaMKII prolongs signaling at the synapse inducing a signal to the nucleus to activate expression of plasticity-related proteins that are required for late LTP (Redondo & Morris, 2011). Further, T286 autophosphorylation signaling

suppresses MIS generation by preventing mTOR-dependent translation of psd95 mRNA. However, in the absence of T286 autophosphorylation signaling mTOR signaling leads to increased PSD-95 expression at the synapse. PSD-95 binds to nNOS to produce a retrograde signal to attract a presynaptic terminal(s). Moreover, increased PSD-95 expression can lead to formation of a separate PSD in the same spine, which can allocate AMPA and NMDA receptors in the adequate postsynaptic position to establish a new synaptic contact.

The hypothesis that MIS generation is a LTM mechanism after multiple training trials raises questions for further investigation, such as: (1) Is MIS generation associated with impaired LTM formation and updating in normal, wild-type animals under conditions when LTP is compromised? For example, normal ageing is associated with impaired NMDA receptor-dependent LTP at CA1 synapses (Bach et al., 1999; Murphy et al., 2004). Thus, is age-associated LTM impairment caused by MIS generation? (2) Does abnormal MIS generation cause deficits in LTM formation

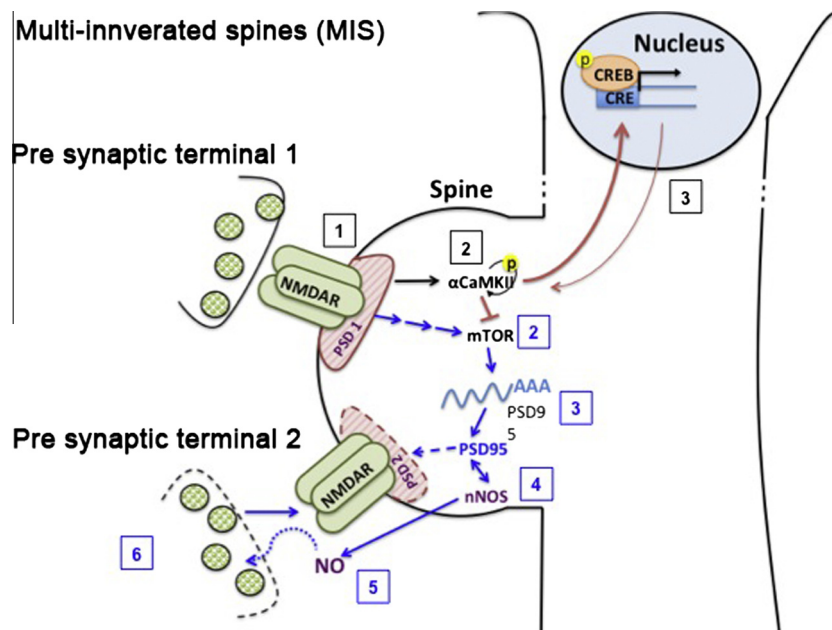


Fig. 2. Signaling mechanisms showing crosstalk between LTP induction and MIS generation. Normally, NMDA receptor activation leads to T286 autophosphorylation of αCaMKII that induces synthesis of plasticity-related proteins for late LTP and additionally blocks activation of mTOR signaling (1–3). Without T286 autophosphorylation multiple NMDA receptor activations can induce mTOR signaling to locally synthesize PSD-95 protein. PSD-95 binds to nNOS to produce the retrograde messenger NO to attract a presynaptic terminal onto the existing dendritic spine to generate a MIS (2–6).

and updating in diseases that impair LTP, such as the early stages of Alzheimer's disease (Sheng, Sabatini, & Sudhof, 2012)? (3) Is the suppression of MIS generation dismissed in models of autism, so that next to LTP MIS are generated and cause deficits in LTM formation?

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